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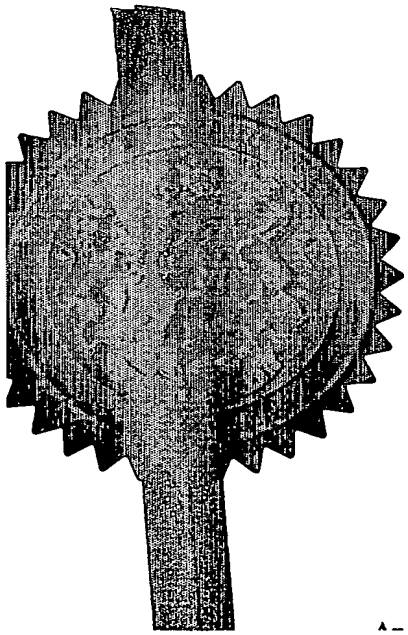

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   Robert Peter MILLAR
   20a West Bay Road
   North Berwick
   EH39 4AW

   Patents ADP number *(if you know it)* 08508809001

   If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — "Vaccine"

5. Name of your agent *(if you have one)* — Murgitroyd & Company

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Vaccine

The invention relates to the use of a retro-inverso peptide of GnRH as a vaccine with therapeutic applications in sex disorders, contraception and steroid sensitive cancers.

The decapeptide gonadotropin releasing hormone (GnRH) regulates the reproductive hormone cascade by stimulating the release of gonadotropins from the anterior pituitary, which in turn regulates reproductive function.

GnRH is synthesised in the neurones of the hypothalamus and released into the portal circulation where it interacts with GnRH receptors on the gonadotrope cells in the anterior pituitary [6]. Stimulation of the GnRH receptor is essential for the secretion of luteininzing hormone (LH) and follicle stimulating hormone (FSH), which in turn are required for steroidogenesis and gametogenesis, respectively [6]. Due to this central role in

reproduction, GnRH peptide analogs have found therapeutic applications in controlling fertility, cryptorchidism, polycystic ovarian syndrome, leiomyomata, endometrioses, acute intermittent porphyria, and breast, ovarian and prostatic cancer [3, 16].

Although a variety of contraceptive methods are available to control fertility, each has disadvantages such as affordability, application difficulty (injections), daily intake (pills) and irreversible procedures (surgical methods). There is a demand for improved and a cost-effective approach to regulate reproductive function, of which immunoneutralisation of GnRH with synthetic peptides has proved successful [7]. Peptide based vaccines against L-amino acid native GnRH conjugates have been shown to be effective in regulating fertility in animals [1, 17] and have subsequently undergone clinical trials in treatment of prostate cancer in humans [22] and have potential in sex hormone-dependent male and female cancers [8, 21]. The advantages of peptide based vaccines are well described as they are chemically defined, are indefinitely stable and can be stored as a freeze dried powder. The preparation does not require large-scale production and is relatively cheap. However, a major limitation of peptide vaccines is their relatively low immunogenicity and limited biological half-life [20].

In RI-peptides the residues are aligned in the reverse order of that in the parent peptide and D-amino acids replace the L-amino acids making RI-peptides powerful Immunogens [25]. The orientation of the side chains in a RI-analog is very similar to that in the parent peptide, which leads to eliciting antibodies that cross-react strongly with the parent L-structure [19;2a;2b]. RI-peptides are protease resistant and induce longer lasting immune responses and high titres of antibodies than do L-peptides [25].. Their resistance to proteolytic enzymes suggests that they may have oral activity. In addition, antibodies to RI-peptides often have greater affinity than antibodies induced to an antigenic site of foot-and-mouth disease virus structure [25;19].

Although GnRH vaccines are employed in a variety of sex-hormone dependent disorders and contraception, their peptide nature has necessitated administration by means of injection. The development of a potent immunogenic non-peptide orally active GnRH vaccine would therefore greatly enhance the utility of this important pharmaceutic agent in current therapies.

Retro-inverso (RI) peptides are peptides where the amino-acid residues are aligned in the reverse order of that in the parent peptide and D-amino acids replace the L-amino acids making the RI-peptides powerful immunogens [25]. The orientation of the side chains in a RI-analog is very similar

to that in the parent peptide, which leads to eliciting antibodies that cross-react strongly with the parent L-structure [2a, 2b, 19]. RI-peptides are protease resistant and induce longer lasting immune responses and high titres of antibodies than do L-peptides [19]. Sometimes antibodies to RI-peptides may have greater affinity than antibodies to classical L-peptides, and show strong neutralising activity as seen in the case of antibodies induced to an antigenic site of foot-and-mouth disease virus [25, 19].

Statement of the Invention

It has now been demonstrated that a retro-inverso GnRH peptide can be used as a vaccine in a mammal, in order to elicit an immune response directed against GnRH.

RI-GnRH peptide has several advantages over classical vaccination methods, as they are highly immunogenic and specific. Additionally, it can be absorbed orally and be therefore more practical especially for use as domestic or companion animal contraceptives, animal husbandry, and controlling wild life populations. In human it offers some potential as contraceptive agents and in the treatment of sex hormone dependant cancers.

The invention thus relates to a retro-inverso (RI) GnRH peptide comprising the sequence GPRLGYSWHE (all D-amino acids). Each letter in the sequence

GPRLGYSWHE refers to a specific amino acid, according to the standard one letter amino acid code. Thus, G represents the amino acid glycine, for example. Advantageously an additional D-cysteine residue is added at the C-terminus for conjugation purposes, and hence the sequence would be GPRLGYSWHEC.

The invention also relates to a vaccine which comprises an effective amount of the above described RI-peptide, preferably in association with a pharmaceutically acceptable carrier or excipient.

By "effective amount" we refer to an amount which is sufficient to cause a sufficient specific immune response against GnRH when administered to a mammal. More particularly we refer to the ability of the peptide to cause the production of antibodies able to bind specifically to endogenous GnRH, thus preventing its action.

According to a preferred embodiment, the vaccine may be used as a contraceptive vaccine to elicit an immune response against GnRH in a mammal sufficient to inhibit or substantially reduce the fertility of said mammal.

According to a particular aspect of the invention one or more adjuvants might be used in association with the RI-GnRH peptide to enhnance its efficiency. Suitable adjuvants can be CpGs, M59,

IFA (incomplete Freund adjuvant), alum, alternated toxins (e.g. pertussis and cholera) etc... The particular adjuvants depends upon the specific species targeted to be treated and the mode of administration chosen.

According to a particularly preferred aspect of the invention, the RI-peptide is administered orally.

In this case the RI-peptide may be conjugated to suitable agents like bile salts, alternated toxins (e.g. pertussis and cholera) and/or activity absorbed vitamins in order to facilitate absorption across the gastro-intestinal tract.

In preferred embodiment the RI-GnRH peptide of the invention is not chemically linked or conjugated with another compound like a carrier or an adjuvant. This allows simplifying and diminishing the cost of the manufacture of the formulation.

The invention further relates to a method of vaccination against GnRH which method includes the step of administering to a mammal an amount of the abovementioned RI-peptide sufficient to elicit an immune response.

The invention further relates to a method of contraception which method includes the step of administering to a mammal a contraceptively effective amount of the abovementioned RI-peptide.

The invention further relates to the use of the abovementioned RI-peptide to elicit an immune response, preferably a contraceptively effective immune response, in a mammal against GnRH.

The invention further relates to the use of the abovementioned RI-peptide as a contraceptive agent in mammals.

The invention further relates to the use of a RI-peptide as described above in the manufacture of a medicament or vaccine, especially in the manufacture of a contraceptive drug, to cause an immune response against GnRH.

Description of the Drawings

Fig. 1 Titre of affinity with RI-GnRH MOA conjugate of purified fractions of anti-RI-GnRH antibodies from one of the rabbits immunised with RI-GnRH MOA conjugate. The fractions were eluted with KSCN ( ), glycine (•), acetic acid-NaCl (•), G-HCl (•). The binding of whole serum () and column flow through (•) is also shown.

Fig. 2a Rabbit Anti-RI-GnRH antibodies cross-react with RI-GnRH peptide with high specificity. RI-GnRH peptide was chemically fixed to ELISA plates. The amount of purified anti-RI-GnRH antibodies, which could bind to the plate,

was suppressed by adding free RI-GnRH peptide to this reaction as described below. Antibodies eluted by various chaotropic agents are indicated by symbols. KSCN1 (•), KSCN2 (·), glycine (•), acetic acid-NaCl (∇) and G-HCl ( ). A non-related peptide (V9C, •) showed low inhibition of binding of the KSCN antibody fraction to RI-GnRH.

Fig. 2b Rabbit Anti-RI-GnRH antibody fractions discriminate GnRH from related analogues. RI-GnRH peptide was chemically fixed to ELISA plates. The graph shows that for the various elutions (with KSCN and glycine, acetic acid-NaCl and G-HCl) the amount of purified anti-RI-GnRH antibodies which could bind to the fixed RI-peptide was suppressed by adding free GnRH (•), [Gln^8]-GnRH(CIGnRH) (•) and GnRH II (CII).

Fig. 3 Binding kinetics with BIAcore of two anti-RI-GnRH antibody fractions. RI-GnRH peptide was immobilised to the sensor chip. The amount of anti-RI-GnRH antibody binding to RI-GnRH peptide was suppressed by adding increasing concentrations of RI-GnRH-peptide (a) or GnRH (b). Two **eluted** antibody fractions were tested, KSCN (•) and G-HCl ( ).

Fig. 4a Immunised rabbit serum inhibits GnRH stimulated IP accumulation. Effect of **preimmune** serum (S) from rabbits before immunisation on GnRH (1nM and 10nM) accumulation in COS-1 cells transiently transfected with human GnRH receptor (top panel). Inhibition of GnRH (1nM and 10nM) stimulated IP accumulation with serum collected from rabbits immunised with RI-GnRH (bottom panel).

Fig. 4b Rabbit anti-RI-GnRH antibodies selectively inhibits GnRH stimulated IP accumulation. Anti-RI-GnRH antibodies **eluted from the affinity column with different agents** were preincubated with GnRH, **cGnRH I** and GnRH II. This cocktail was added to cells transfected with the human GnRH receptor as described **(solid columns)**. The effect on ligand stimulated IP accumulation was calculated as a percentage inhibition of ligand stimulated IP accumulation in the absence of antibody (open columns).

Fig. 4c Dose-response curves of GnRH stimulated IP accumulation was suppressed by rabbit anti-RI-GnRH antibodies. COS-1 cells were transiently transfected with the human GnRH receptor and GnRH stimulated IP accumulation was measured. The EC_{50} of GnRH (0.25nM) was suppressed when 5nM anti-RI-GnRH antibodies of KSCN (3.6 and

1.5nM) and glycine (1.1nM) fractions were pre-incubated with GnRH.

Fig. 4d Effect of increasing doses of rabbit anti-RI-GnRH antibody fractions on GnRH (0.3nM) stimulation of IP accumulation over 1 hour. GnRH was preincubated with varying concentrations of antibody fractions.

Fig. 5a Titre of anti-RI-GnRH antibodies raised in five male mice (M1-M5) immunised with RI-GnRH conjugated to MAO in CFA. Control mice (M6-M9) received only methylated BSA in CFA. Methylated BSA in CFA was used with RI-GnRH in the booster injections for test animals.

Fig. 5b Inhibition of binding of mouse anti-RI-GnRH sera to RI-GnRH by increasing concentrations of m-GnRH in experiment 1. RI-GnRH was immobilised on ELISA plates and incubated with serum collected on day 22 after initial mating in the presence of increasing concentrations of native GnRH.

Fig. 6a Immunising male mice with RI-GnRH peptide decreases their fertility in the first mice experiment. Relationship between anti-RI-GnRH affinity for GnRH and litter size of normal females mated with immunised male mice.

Fig. 6b Relationship between testis weight of immunised male mice and litter size of normal females mated with them in the first mice experiment.

Fig. 7 Titre of anti-RI-GnRH sera from mice from the second mice experiment which are immunised with RI-GnRH and CpG but without FCA. M1, M3 and M4 are males, M2, M5 and M6 were females.

Fig. 8 Second mice experiment: Relationship between anti-RI-GnRH titre and litter size resulting from pairing of RI-GnRH CpG immunised male (M1, M3, M4) and female (M2, M5, M6) mice with normal partners. Solid columns are titre, open columns are litter size.

Materials and Methods

All the peroxidase conjugated second antibodies came from Jackson, (Pennsylvania, USA) and Malemide activated Ovalbumin (MAO) from Pierce Illinois, USA. Methylated Bovine Serum Albumin (m-BSA) was obtained from Calbiochem and tissue culture medium from Life Technology (Cergy Pontoise, France) and COS-1 cells from ATCC (USA). Chromatography columns and Sepharose beads came from Pharmacia (Uppsala, Sweden). CpG oligonucleotide was synthesised by Eurogenteche (Brussels, Belgium). Mammalian GnRH (GnRH), chicken GnRHI ([Gln^8]-

GnRH/cGnRH I) and GnRHII were prepared by conventional solid phase methodology and purified by preparative C-18 reverse phase HPLC (University of Cape Town Laboratory) and myo[2-^{3}H]-inositol was purchased from Amersham (United Kingdom).

Peptide Immunogen

A retro-inverso peptide corresponding to GnRH (RI-GnRH) was synthesised by using Fmoc technology and was purified by HPLC and checked with mass spectrometry. The sequence of the RI-GnRH peptide was GPRLGYSWHEC (all D-amino acids) which included the additional cysteine residues at the C-terminus for conjugation purposes.

Peptide Conjugation

The day before primary injection RI-GnRH was mixed with malemide-activated ovalbumin (MAO), and incubated for one hour at room temperature. It was then dialysed over night against PBS (10mM phosphate, 140mM NaCl, pH = 7.4).

Immunisation

Rabbit Experiment

Two adult female rabbits (Harlan, Leicestershire, UK) were immunised with RI-GnRH (100µg/rabbit) conjugated with MAO. The primary injections were given subcutaneously in Complete Freund's Adjuvant

(CFA). These were followed by three booster injections in Incomplete Freund's Adjuvant (IFA) at two week intervals. The last booster injection was given four weeks later, using 200µg free peptide together with 1mg methylated-BSA. The rabbits were bled one week after each injection.

First Mice Experiment (Experiment 1)

Male Balb/c mice (Janvier, France), nine weeks of age (n=9), were immunised intraperitoneally (i.p) with either 25µg/mouse of RI-GnRH conjugated with MAO (n=5) or with saline buffer (n=4). The primary injections were given in CFA supplemented with 200µg m-BSA. These were followed by two-booster injections In IFA and m-BSA at day 15 and 45 after primary injections. The mice were weighed and bled one week after each immunisation. On day 45, males injected with RI-GnRH conjugate or saline were placed with females of proven fertility and litters observed. The males were sacrificed 75 days after primary injection for histological examination.

Second Mice Experiment (Experiment 2)

In a second experiment, four week old Balb/c mice (3 of each sex) were immunised with 25µg/mouse of unconjugated RI-GnRH. A control group received saline buffer together with 50µg/mouse of a CpG oligonucleotide as adjuvant (Klinman, et al., 1999) supplemented with 200µg of m-BSA (2 of each sex).

All injections were given in 10% v/v IFA. Mice were immunised at days 1, 15 and 30. They were bled and mated with partners of proven fertility at day 37 after the initial immunisation.

Enzyme Immunoassay (ELISA)

Microtitre plates were coated with RI-GnRH peptide (5µg/ml) in 100mM Na_2CO_3 (pH = 9.6) and incubated for one hour at 37°C. After several washes with PBS (pH = 7.4) plates were saturated for one hour with 1% BSA in PBS supplemented with Tween 20 (0.1% wt/volume), at 37°C. Sera from immunised rabbits and mice at different dilutions (1/500 to 1/3200) were added to the plates and incubated for one hour at 37°C. Plates were washed several times and allowed to react with peroxidase conjugated Goat anti-rabbit or anti-mouse (affinity purified Fc specific IgG) for one hour at 37°C. Washed plates were reacted with 3,3'5,5'-tetramethyl benzidine (TMB) and hydrogen peroxidase as substrate.

Inhibition Immunoassay

Before using the sera in the enzyme immunoassay, various dilutions of serum samples were preincubated with increasing concentrations (3.65pM to 20nM) mammalian GnRH (GnRH) for one hour at 37°C.

Antibody Purification

Sepharose 4B beads (1mg) with activated thiol groups was used to couple 4 mM of RI-GnRH according to the standard procedures. Sera from a rabbit immunised with RI-GnRH was precipitated with saturated ammonium sulphate solution (40%) and dialysed over night at 4°C against PBS (pH = 7.4). Immunoglobulins were diluted 15 times to a final concentration of 10mg/ml and passed through the column (100µl/min) for three hours at 4°C. The anti-RI-GnRH antibodies were successively eluted with potassium thiocyanate (3M KSCN), glycine (2M pH = 2.8), acetic acid (CH_3COOH and NaCl, pH = 2.1) and guanidium hydrochloride (6M G-HCl). Two fractions of anti-RI-GnRH antibodies were eluted with potassium thiocyanate (KSCN 1 and 2) and one fraction for each of the other chaotropic agents. All the fractions were immediately dialysed over night at 4°C against PBS.

Surface Plasmon Resonance (SPR)

RI-GnRH peptide was fixed on a sensor chip (Pharmacia biotech, Uppsala, Sweden) by the standard thiol immobilisation on protocol using the upgraded BIA 1000 (Pharmacia Biotech, Uppsala, Sweden). Affinity purified rabbit anti-RI-GnRH antibodies were injected with a flow of 5µl/min at the total volume of 100µl, over the sensor chip. Anti-RI-GnRH antibodies (50nM) were preincubated

either with RI-GnRH (7.8-500nM) or GnRH (7.8-1000nm) for 15 mins at room temperature, and were injected under the same condition as above. The sensorgrams were recorded and analysed by BiaEvaluation 3 Pharmacia Biotech, Uppsala, Sweden).

Transfection and Cell Culture

COS-1 cells were cultured in Dulbecco's modified Eagle's medium/DMEM (Gibco, Paisley, Scotland), supplemented with 10% foetal calf serum (FCS, Delta bioproducts, Kempton Park, South Africa) in a 10% CO_2 incubator at 37°C. Cells were harvested with 0.05% trypsin. For all transient transfections 2.10^5 cells/well were seeded into 12-well plates and cultured overnight in DMEM containing 10% FCS and antibiotics (2mg/ml streptomycin sulphate, 4000U/ml sodium benzylpenicilin). COS-1 cells were transiently transfected using the DEAE-Dextran method [10], as previously described [15].

Phosphatidyl Inositol hydrolysis

The transfected COS-1 cells ($2x10^5$ cells/well) were incubated overnight in 0.5ml Medium 199 (Gibco, Paisley, Scotland) with antibiotics and *myo*-[2-^{3}H] inositol (1µCi/well, Amersham, Arlington Heights, England) as previously described [15]. The labelled cells were incubated with various concentrations of GnRH-analogues for one hour at 37°C in the presence of LiCl as described [15].

(10^{-6} to 10^{-10} M) for two hours at 37°C in buffer. The mixture was added to labelled cells as described and the inhibition of IP production calculated. Additionally, increasing concentrations of purified anti-RI-GnRH antibodies (0.1-5nM) were preincubated with 0.3nM of GnRH for two hours at 37°C in buffer. The mixture was added to labelled cells and the inhbition of IP production calculated as described.

Histology

Male mice were sacrificed and the testes dissected. Testes were weighed and fixed in formalin/saline solution (12%) for routine histology. The fixed testes were dehydrated, sectioned (5µm thick) and stained with hematoxylin and eosin.

RESULTS

Immunised rabbits develop antibodies against RI-GnRH and GnRH

Both immunised rabbits produced high titre anti-RI-GnRH polyclonal antibodies. As shown in Figure 1 we were able to detect and purify different populations of antibodies with different affinity for RI-GnRH peptide and native GnRH. Antibody populations eluted by chaotropic agents such as acid glycine, CH3COOH-NaCl and G-HCl recognised the RI-GnRH peptide with higher affinity than those

eluted with KSCN. This shows the heterogenous nature of the raised polyclonal antibodies.

Anti-RI-GnRH antibodies in all eluted fractions bound the fixed RI-GnRH peptide on ELISA plates. Increasing amounts of free RI-GnRH peptide incrementally decreased the amount of anti-RI-GnRH antibodies available to bind the fixed RI-GnRH peptide on ELISA plates (Fig. 2a). The anti-RI-GnRH antibody binding was not significantly inhibited by unrelated L-peptide sequence, VRTVEDGEC (V9C). This suggests that these antibodies bind the RI-GnRH-peptide sequence with high specificity (Fig. 2a).

Native GnRH could also inhibit the different eluted fractions of anti-RI-GnRH antibodies from binding to the immobilised RI-peptide (Fig. 2b). This demonstrates that the anti-RI-GnRH antibodies cross-react with the parent L-amino acid sequence. Of the entire set of eluted antibody fractions, the KSCN fractions cross-reacted most efficiently with GnRH (Fig. 2b). This suggests that the KSCN eluted fraction of antibodies have the highest affinity for GnRH. The antibodies cross-reacted with GnRH with higher affinity compared with two GnRH related analogs (cGnRH I and GnRH II). These analogues have one ([Gln^8]-GnRH) and three ([$His^5Trp^7Tyr^8$]-GnRH) amino acid substitutions, respectively. This demonstrates that anti-RI-GnRH antibodies discriminate GnRH from related isoforms (Fig. 2b).

Binding kinetics of two anti-RI-GnRH antibody fractions

The antibody fraction eluted with G-HCL had the higher affinity for RI-GnRH ($Ka=5,38.10^{8}\ M^{-1}$; $Kd=1,86.10^{-9}M$) than the KSCN1 fraction ($Ka=2,89.10^{3}M^{-1}$; $Kd=2.34.10^{-4}M$). The amount of anti-RI-GnRH antibody binding to RI-GnRH peptide immobilised on the sensor chip was inhibited more by free RI-GnRH preincubated with the G-HCL antibody fraction than with the KSCN1 antibody fraction (Fig. 3a). This suggests that the G-HCl eluted antibody fraction is most specific for RI-GnRH peptides. Conversely, GnRH could suppress the KSCN1 antibody fraction from binding the RI-GnRH peptide (immobilised on the sensor chip) more effectively than the G-HCl antibody fraction (Fig. 3b). This indicates that the KSCN1 fraction has the highest antigenic cross-reactivity with GnRH.

Anti-RI-GnRH antibodies inhibit GnRH stimulated inositol phosphate accumulation

Serum collected from rabbits same "2" rabbits immunised with RI-GnRH peptide could inhibit GnRH stimulated inositol phosphate (IP) accumulation in COS-1 cells transiently transfected with human GnRH receptor (Fig. 4a). Serum from the same rabbits before immunisation had no effect on GnRH stimulated IP accumulation.

The ability of the different anti-RI-GnRH antibody eluted fractions to inhibit GnRH, cGnRH I and GnRH II stimulated IP accumulation was compared (Fig. 4b). The KSCN eluted antibody fractions were the most effective in inhibiting GnRH stimulated IP accumulation than was the glycine eluted antibody fraction. The CH_3COOH-NaCl and G-HCl eluted antibody fractions did not inhibit GnRH stimulated IP production. This suggests that KSCN eluted antibody fractions have the highest level of antigenic cross-reactivity with the parent L-peptide as observed in the binding studies. Additionally, the RI-GnRH peptide alone could not stimulate or inhibit GnRH stimulated IP accumulation (data not shown). This shows that the RI-GnRH peptide would have no additional effect on GnRH function, other than acting as an immunogen raising neutralising GnRH antibodies.

The anti-RI-GnRH antibodies eluted with KSCN and glycine inhibited GnRH stimulated IP accumulation over a range of GnRH concentrations (Fig. 4c). The EC_{50} values calculated (Prism, GraphPad Software Inc., San Diego) from dose-response curves of GnRH stimulated IP accumulation was suppressed by the KSCN (15- and 6-fold) and glycine (4.5-fold) antibody fractions. Increasing concentrations of antibody fractions from KCSN, glycine and CH3COOH-NaCl elutions, progressively inhibited 0.3nM GnRH stimulated IP accumulation (Fig. 4d). Most antibody fractions eluted with KSCN1 elution was not effective and inhibited GnRH (0.3nM) stimulated

IP accumulation by >90% (Fig. 4d). The concentration of GnRH in the hypothalamic-hypophyseal portal system is similar to this.

RI-GnRH immunised mice produce antibodies which inhibit reproduction

All five immunised male mice developed anti-RI-GnRH antibodies (Fig. 5a) and the five of highest titre also bound native GnRH (Fig. 5b). Two of the group of five immunised mice was infertile, one mouse fostered one pup and the remaining two had four and five pups respectively. There was a correlation between antibody titre and IC_{50} with the suppression of fertility. The highest affinity for GnRH was detected in the infertile mice while the lowest was in the fertile ones (P=0.008, Fig. 6a). Testis weight was directly related to litter size (P=0.004, Fig. 6b). Mating of control male mice with normal females results in normal litter size (5-7 pups). Testis weight was also directly related to litter size.

In the second experiment, mice immunised with RI-GnRH peptide and CpG as adjuvant produced anti-RI-GnRH antibodies, which cross-reacted the native hormone (Fig. 7). All mice were infertile after pairing at day 37, except for one, which had a delayed immune response to RI-GnRH (Fig. 8). Infertility persisted after the initial mating as there were no litters resulting from further matings up to day 90. The mice were boosted once

more at day 97 and mated one week thereafter (Table 1). Two mice were infertile on day 120, while four stayed infertile until day 220, when they were sacrificed. Infertility was related to the titre of anti-RI-GnRH (Table 1). Control female mice had normal litter size and female partners of control male mice also had normal litter size. In both experiments there was no indication of adverse side effects or changes in body weight of the mice. Histological evaluations of sections of the testes of mice treated with RI-GnRH peptide revealed atrophied Leydig and Sertoli cells, less spermatogonia and primary spermatocytes, presence of very few spermatids and reduced diameters of the seminiferous tubules. These results indicate suppressed spermatogenesis in the immunised infertile mice as shown (Fig. 9a and b).

Discussion

We show that immunising experimental animals with a RI-GnRH peptide elicits polyclonal anti-RI-GnRH antibody production in rabbits and mice, which possess a high level of antigenic cross-reactivity with the parent L-amino acid peptide, GnRH. The level of anti-RI-GnRH antibodies produced in sera was detected with ELISA and both RI-GnRH and native GnRH bound the antibodies. Sera containing anti-RI-GnRH antibodies were able to effectively inhibit GnRH stimulated IP accumulation in COS-1 cells transiently transfected with the human GnRH receptor. The RI-GnRH peptide did not stimulate or

inhibit GnRH stimulated IP accumulation. This suggests that the RI-GnRH peptide would not affect GnRH receptor function, except by immunoneutralisation of endogenous GnRH.

Although RI-peptides have previously been reported to produce antibodies which immunoneutralise native L-amino acid proteins, they have not been employed to immunoneutralise small biologically active peptides such as GnRH. Moreover, the N- and C-termini (pGlu and Gly.NH_2) which are important for binding of GnRH to its cognate receptor cannot be simulated in RI-GnRH. Therefore, it was not predictable that antibodies raised against RI-GnRH, which could bind only the middle region of native GnRH, would immunoneutralise the native peptide.

Antibodies from whole serum were precipitated and the anti-RI-GnRH antibodies were affinity purified and characterised with ELISA and the upgraded BIAcore1000 system [25]. We show that immunising with RI-GnRH peptide produces anti-RI GnRH and GnRH antibodies with varying affinities and specificities. The antibodies with the highest affinity for RI-GnRH peptide had, as expected, the lowest cross-reactivity with the native GnRH while the lower affinity antibodies cross-reacted with native GnRH. Nevertheless the lower affinity antibodies cross-reacted were more selective for mammalian GnRH than other naturally occuring forms (cGnRH I and GnRH II) and are probably the main contributors to the suppression of fertility

observed. The specificity of the antibodies against GnRH was encouraging as most vertebrate species have variant forms of GnRH which are thought to have physiological functions in addition to regulating pituitary hormone release [12;24]. For example, primates have both GnRH and GnRH II, of which GnRH II is predominantly found in extrahypothalmic areas [27;14] and is suggested to have a neuromodulator role [24;9]. The mammalian pituitary GnRH receptor is proposed to discriminate between GnRHrelated peptides [12].

We show that the synthetic RI-GnRH peptide elicited high titres of anti-GnRH antibody and induced sterility in both male and female mice, with no noticeable side affects. Treatment lead to reduced testis weight and low fertilisation and pregnancy rates, which correlated directly with anti-RI-GnRH antibody titre, respectively. Histology of testes revealed atrophied Leydig and Sertoli cells, reduced diameters of the seminiferous tubules and the absence of elongated spermatids in their laminae, confirming the observed suppression of male fertility. These observations are consistent with an inhibition of gonadotropin secretion [13, 18].

It has been demonstrated that a peptide based GnRH vaccine is effective in inducing reversible infertility in humans, which is directly related to the antibody titre [22]. Another study demonstrated that a GnRH vaccine induced

infertility in white-tailed deer lasting up to two years without boosting [17]. Since the RI-GnRH peptide was effective in mice it should have similar results to native GnRH vaccine in other mammals. Since D-amino acid peptides are resistant to proteases it should be active as an oral vaccine.

GnRH vaccines have been suggested to be most practical for use as companion animal contraceptives [13], animal husbandry [1], and controlling wild life populations [17]. Although GnRH vaccines also offer potential as contraceptive agents in humans, concerns over the reversibility and need to supplement sex hormones would have to be addressed. The most likely application in humans would be in the treatment of sex hormone dependant cancers [22].

GnRH vaccines with the RI-GnRH peptide has several advantages over classical vaccination methods. They are highly immunogenic. Additionally, RI-peptides are protease resistant suggesting that they may have oral activity, and if conjugated to bile salts alternated toxins (e.g. pertussis and cholera) and activity absorbed vitamins may facilitate absorption across the gastro-intestinal tract eliciting a specific IgG response.

6. Fink G (1988) Gonadotropin secretion and its control, in *The Physiology of Reproduction.x* (Knobil E and Neill JD, eds.) pp 1349-1377, Raven Press, New York.

7. Ghosh S and Jackson DC (1999) Antigenic and immunogenic properties of totally synthetic peptide-based anti-fertility vaccines. *Int. Immunol* **11**:1103-1110.

8. Jacobs E, Watson SA, Michaeli D, Ellis IO and Robertson JF (1999) Anti-gonadotrophin releasing hormone antibodies inhibit the growth of MCF7 human breast cancer xenografts. *Br J Cancer* **80**:352-359.

9. Jones SW (1987) Chicken II luteininzing hormone-releasing hormone inhibits the M-current of bullfrog sympathetic neurons. *Neurosci Lett* **80**:180-184.

10. Keown WA, Campbell CR and Kucherlapati RS (1990) Methods for introducing DNA into mammalian cells. *Methods Enzymol* **185**:527-537.

11. Klinman DM, et al. (19??). CpG motifs as immune adjuvants. Vaccine 17:19-25.

12. King JA and Millar RP (1995) Evolutionary aspects of gonadotropin-releasing hormone and its receptor. *Cell Mol Neurobiol* **15**:5-23.

13. Ladd A, Tsong YY, Walfield AM and Thau R (1994) Development of an antifertility vaccine for pets based on active immunisation against luteinizing hormone-releasing hormone. *Biol Reprod* **51**:1076-1083

14. Latimer VS, Rodrigues SM, Garyfallou VT, Kohama SG, White RB, Fernald RD and Urbanski HF (2000), Two molecular forms of gonadotropin-releasing hormone (GnRH-I and GnRH-II) are expressed by two separate populations of cells in the rhesus macaque hypothalamus. *Brain Res Mol Brain Res* **75**:287-292.

15. Millar RP, Davidson J, Flanagan CA and Wakefield I (1995), Ligand binding and second messenger assays for cloned Gq/G11-coupled neuropeptide receptors; the GnRH receptor, in *Methods in Neurosciences, Receptor Molecular Biology* (Sealfon SC, ed.) pp 145-162, Academic press, San Diego.

16. Millar RP, King JA, Davidson JS and Milton RC (1987) Gonadotropin-releasing hormone—diversity of functions and clinical applications. *S Afr Med J* **72**:748-755.

17. Miller LA, Johns BE and Killian GJ (2000) Immunocontraception of white-tailed deer with GnRH vaccine. *Am J Reprod Immunol* **44**:266-274.

18. Moudgal N, et al. Development of male contraceptive vaccine-a perspective. Human Reproduction Update 1997, vol. 3, No. 4, pp 335-346.

19. Muller S, et al. (1995) Pept.Res.**8**:138-144.

20. Muller S, et al. (1998) The potential of retro-inverso peptides as synthetic vaccines. Exp.Opin.Invest.Drugs **7(9)**:1429-1438.

21. Talwar GP (1999) Vaccines and passive immunological approaches for the control of fertility and hormone-dependent cancers. *Immunol Rev* **171**:173-192.

22. Talwar GP (1997) Vaccines for control of fertility and hormone dependent-cancers. *Immunol Cell Biol* **75**:184-189.

23. Talwar G (1997). Fertility regulating and immunotherapeutic vaccines reaching human trials stage. Human Reproduction Update (1997), vol. 3, No. 4, pp 301-310.

24. Troskie B, King JA, Millar RP, Peng YY, Kim J, Figueras H and Illing N (1997) Chicken GnRH II-like peptides and a GnRH receptor selective for chicken GnRH II in amphibian sympathetic ganglia. *Neuroendocrinology* **65**;396-402.

25. Van Regenmortel M, et al. Measurement of antigen-antibody interactions with biosensors. J.Mol.Recoqn. 11, 163-167, 1998.

26. Van Regenmortel M, (1997). In Vaccines 97, Cold Spring Harbor Laboratory Press, pp 9-15.

27. White RB, Eisen JA, Kasten TL and Fernald RD (1998) Second gene for gonadotropin-releasing hormone in humans. *Proc Natl Acad Sci U S A* **95**:305-309.

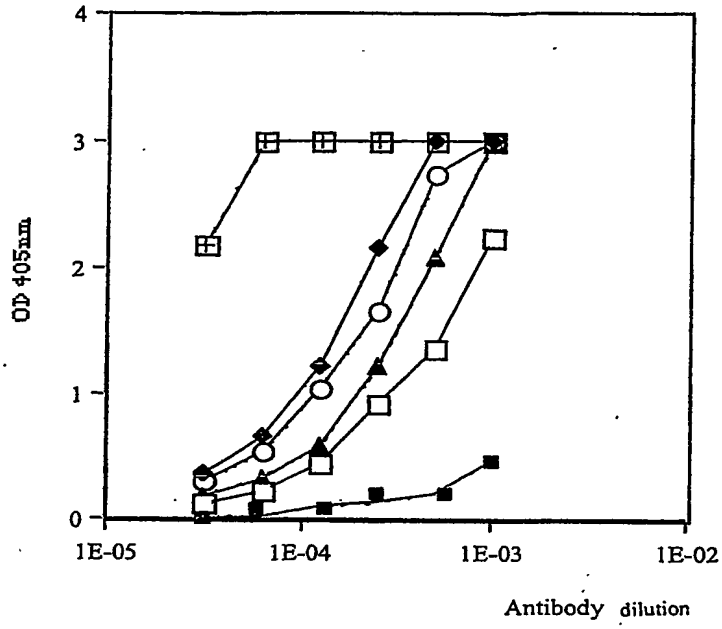


Fig. 1

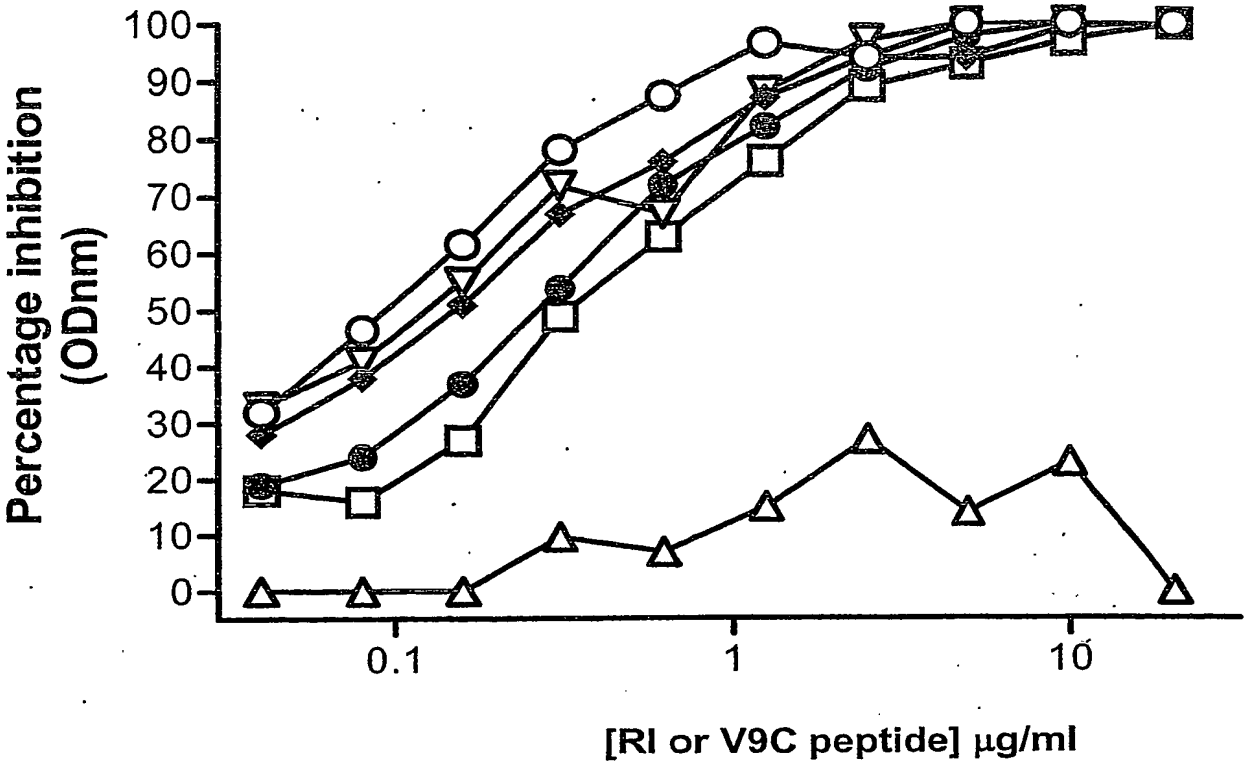
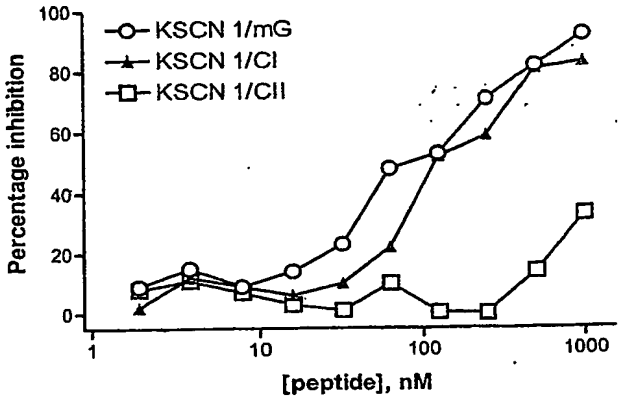
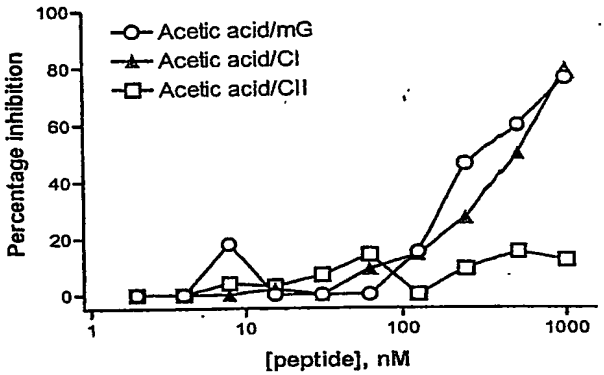
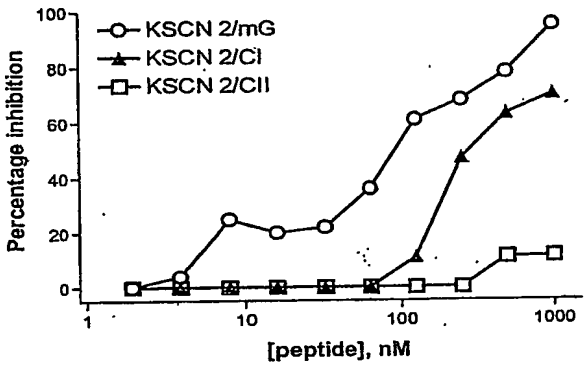
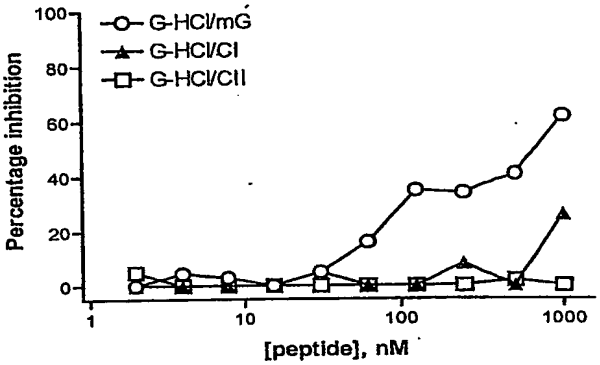

Percentage inhibition (ODnm)
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
[RI or V9C peptide] µg/ml
KSCN 1
KSCN 2
Glycine
Acetic acid
G-HCl
KSCN 1/V9C

Fig. 2a


KSCN 1/mG
KSCN 1/Cl
KSCN 1/ClI
Percentage inhibition
[peptide], nM


Acetic acid/mG
Acetic acid/Cl
Acetic acid/ClI
Percentage inhibition
[peptide], nM


KSCN 2/mG
KSCN 2/Cl
KSCN 2/ClI
Percentage inhibition
[peptide], nM


G-HCl/mG
G-HCl/Cl
G-HCl/ClI
Percentage inhibition
[peptide], nM

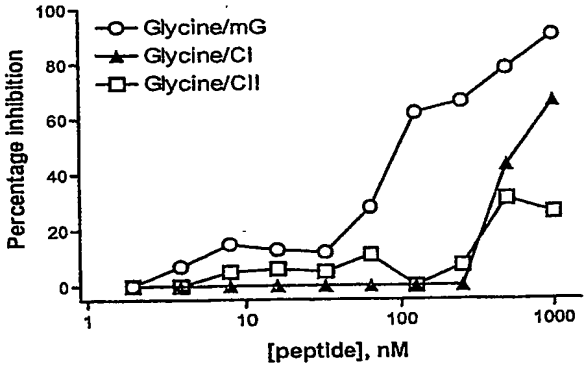

Glycine/mG
Glycine/Cl
Glycine/ClI
Percentage inhibition
[peptide], nM

Fig. 2b

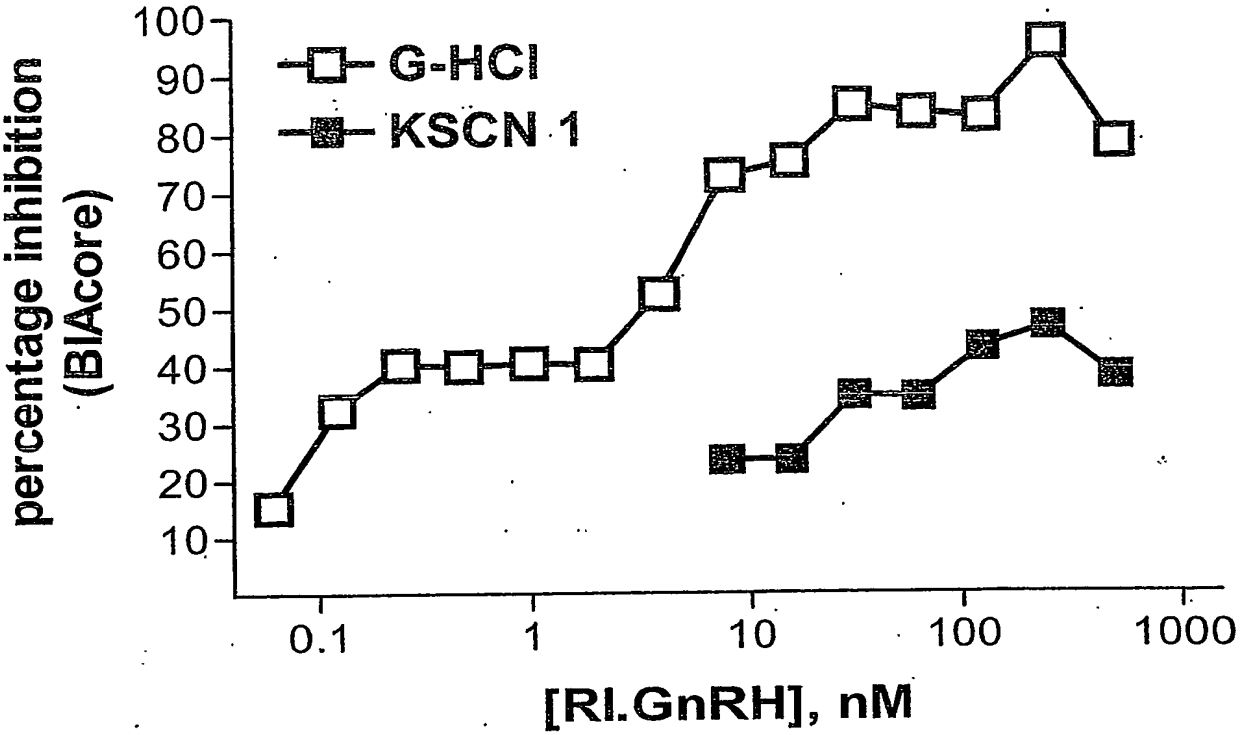

G-HCl
KSCN 1
percentage inhibition (BIAcore)
100
90
80
70
60
50
40
30
20
10
0.1
1
10
100
1000
[RI.GnRH], nM

Fig. 3a

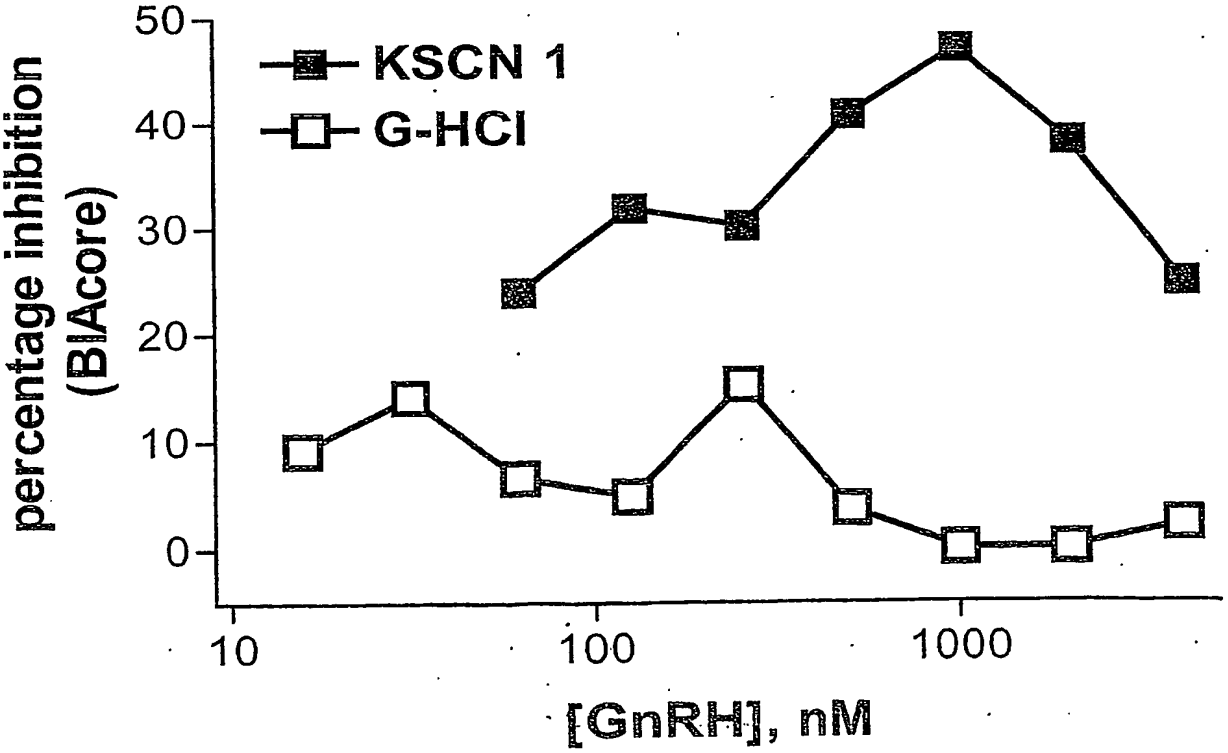

KSCN 1
G-HCl
percentage inhibition (BIAcore)
50
40
30
20
10
0
10
100
1000
[GnRH], nM

Fig. 3b

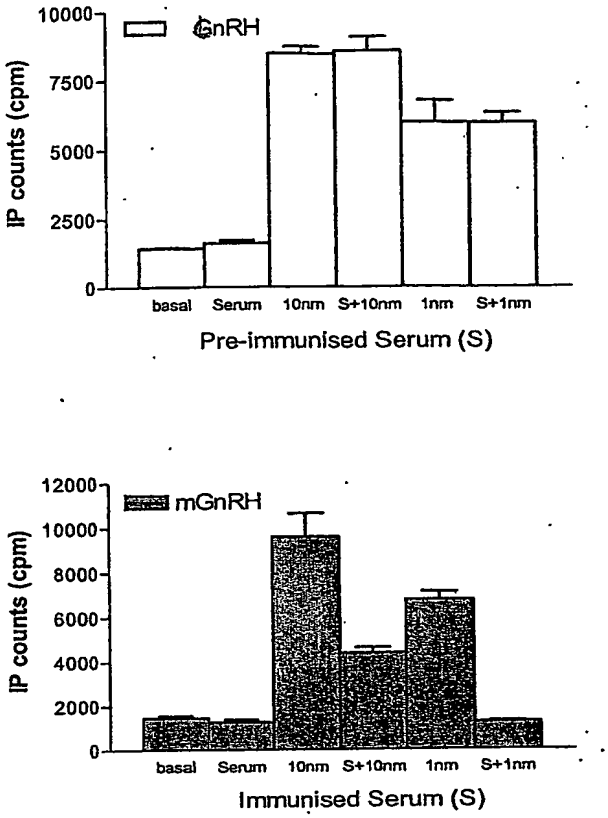

GnRH
IP counts (cpm)
10000
7500
5000
2500
0
basal
Serum
10nm
S+10nm
1nm
S+1nm
Pre-immunised Serum (S)
mGnRH
IP counts (cpm)
12000
10000
8000
6000
4000
2000
0
basal
Serum
10nm
S+10nm
1nm
S+1nm
Immunised Serum (S)

Fig. 4a

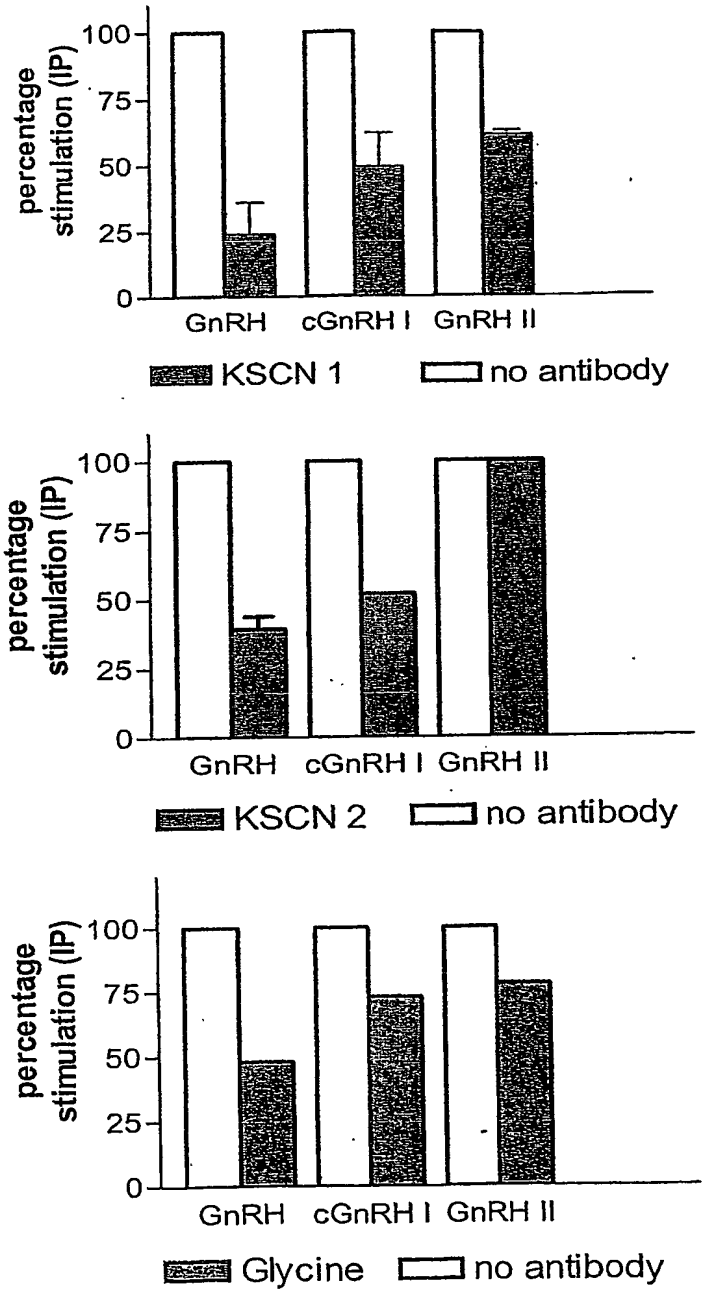

percentage stimulation (IP)
100
75
50
25
0
GnRH
cGnRH I
GnRH II
KSCN 1
no antibody
percentage stimulation (IP)
100
75
50
25
0
GnRH
cGnRH I
GnRH II
KSCN 2
no antibody
percentage stimulation (IP)
100
75
50
25
0
GnRH
cGnRH I
GnRH II
Glycine
no antibody

Fig. 4b

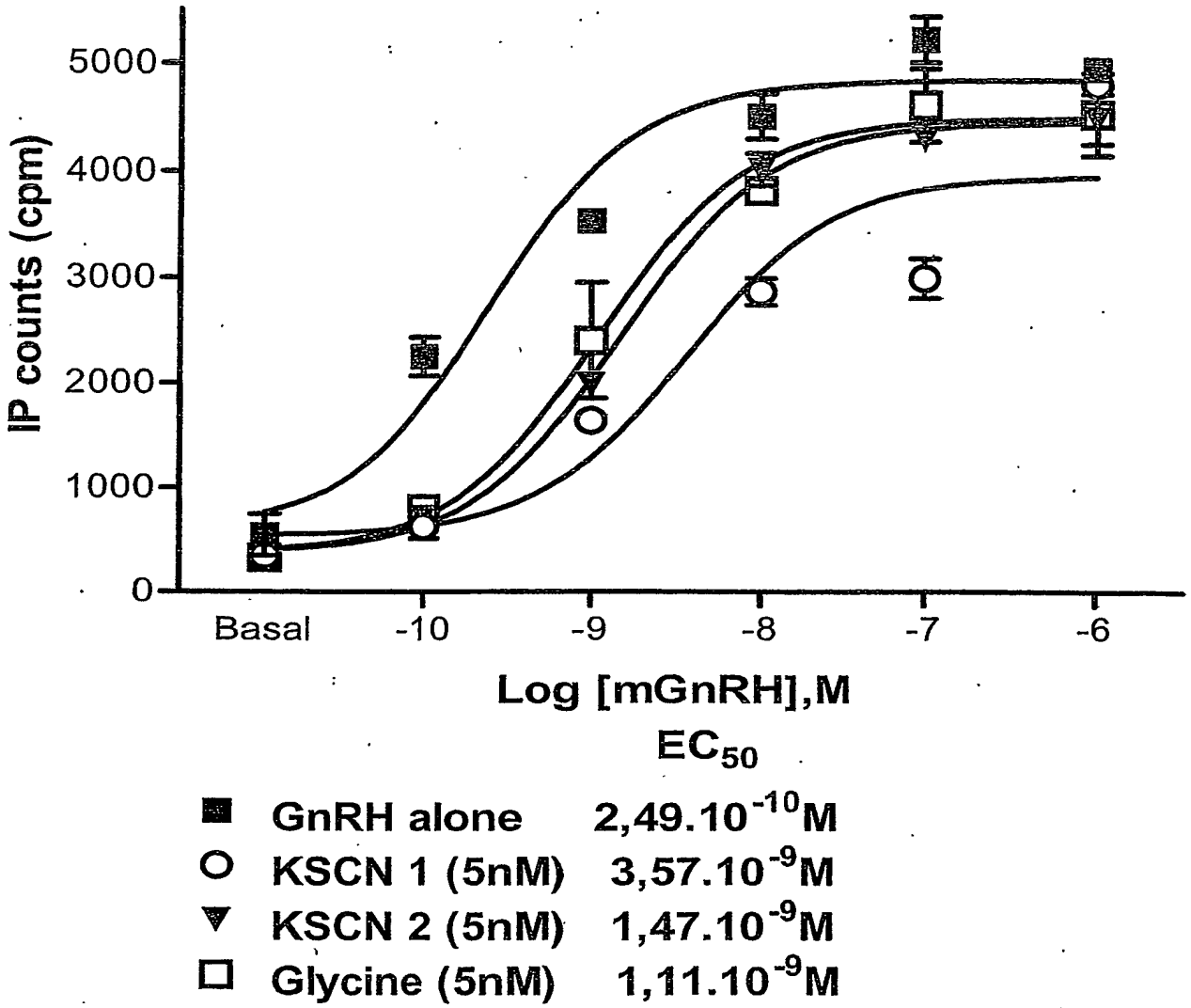
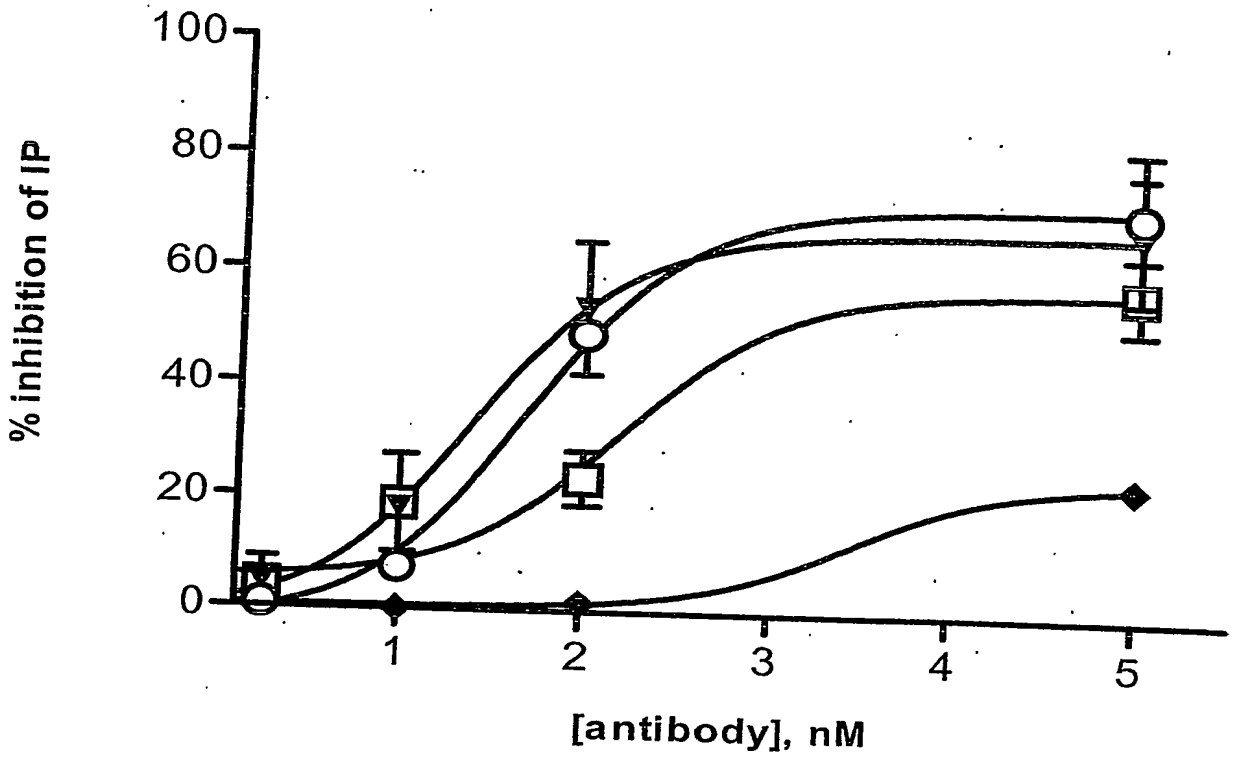

IP counts (cpm)
5000
4000
3000
2000
1000
0
Basal
-10
-9
-8
-7
-6
Log [mGnRH],M
EC50
GnRH alone 2,49.10-10M
KSCN 1 (5nM) 3,57.10-9M
KSCN 2 (5nM) 1,47.10-9M
Glycine (5nM) 1,11.10-9M

Fig. 4c



	EC_{50}
○ KSCN 1	1,7nM
▼ KSCN 2	2,2nM
□ Glycine	3,0nM
◆ Acetic acid	-

Fig. 4d

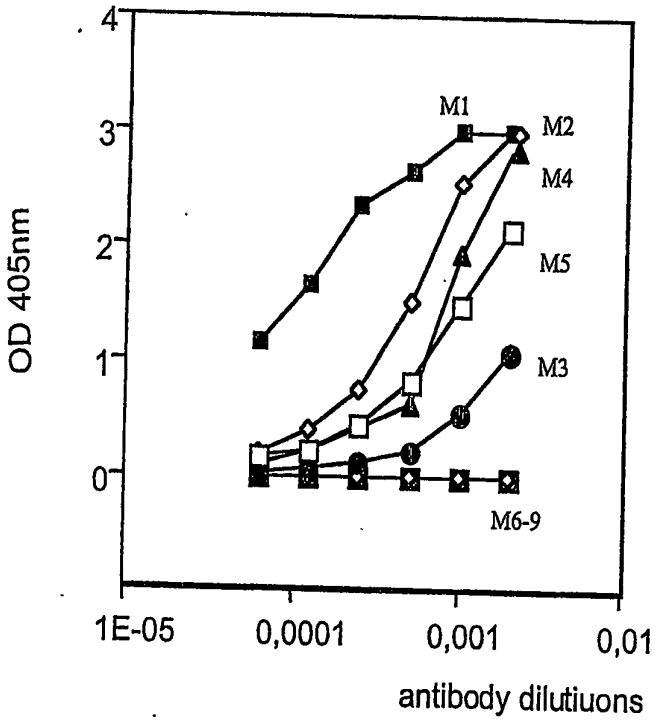

4
3
2
1
0
OD 405nm
M1
M2
M4
M5
M3
M6-9
1E-05
0,0001
0,001
0,01
antibody dilutiuons

Fig. 5a

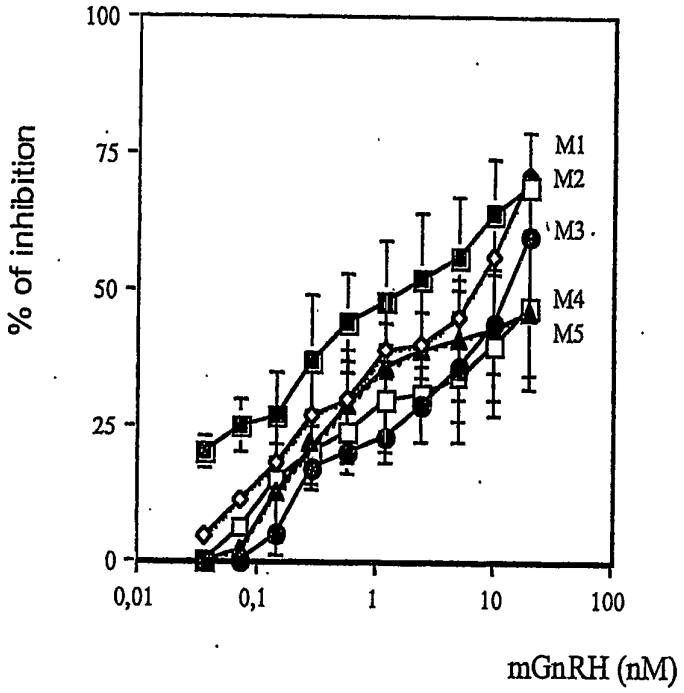

100
75
50
25
0
% of inhibition
M1
M2
M3
M4
M5
0,01
0,1
1
10
100
mGnRH (nM)

Fig. 5b

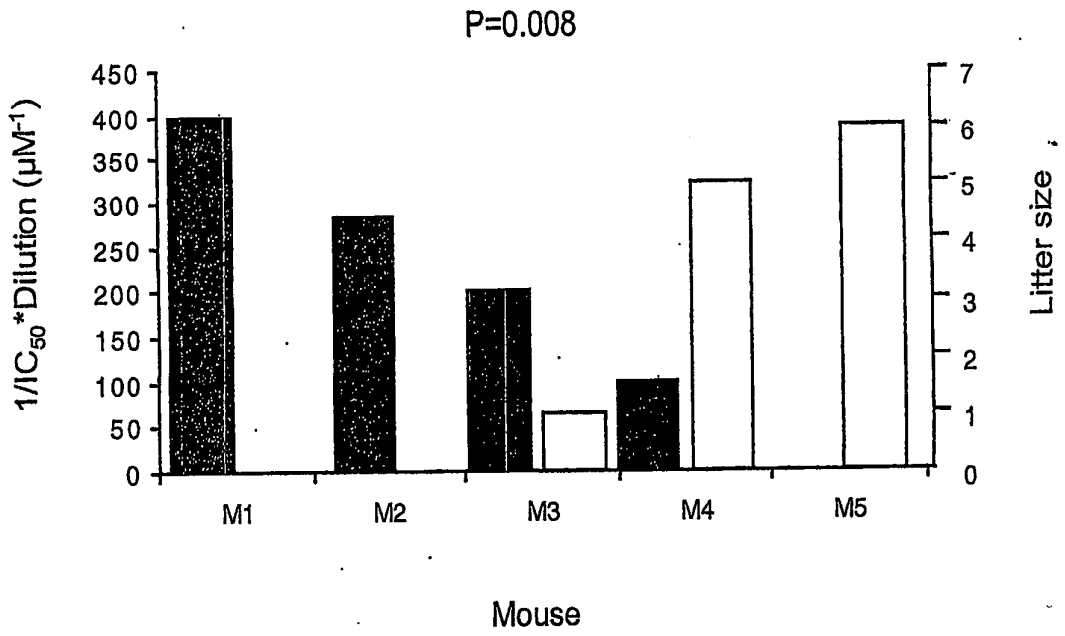

P=0.008
1/IC50*Dilution (μM-1)
450
400
350
300
250
200
150
100
50
0
7
6
5
4
3
2
1
0
Litter size
M1
M2
M3
M4
M5
Mouse

Fig. 6a.

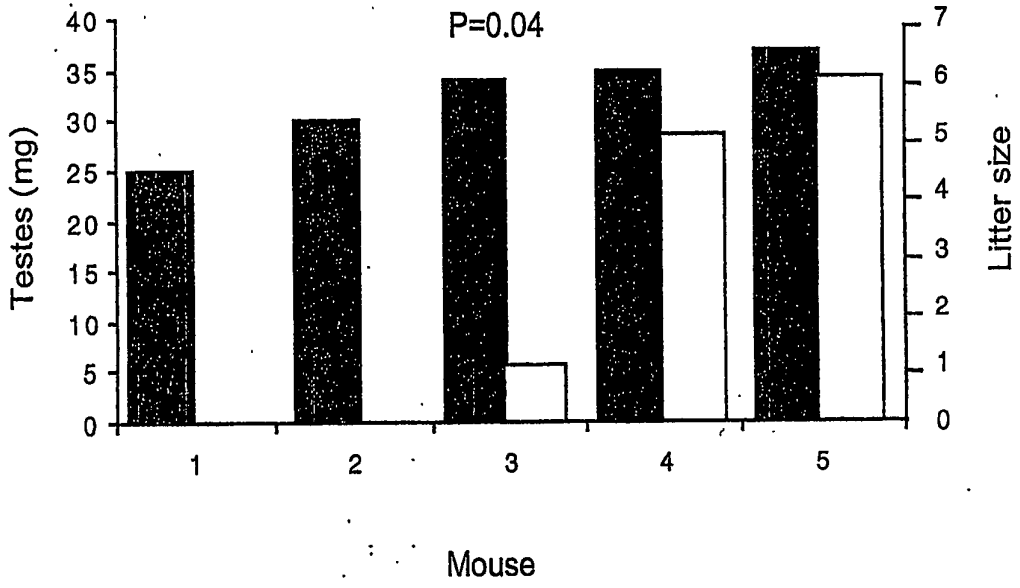

P=0.04
Testes (mg)
Litter size
Mouse
40
35
30
25
20
15
10
5
0
7
6
5
4
3
2
1
0
1
2
3
4
5

Fig. 6b

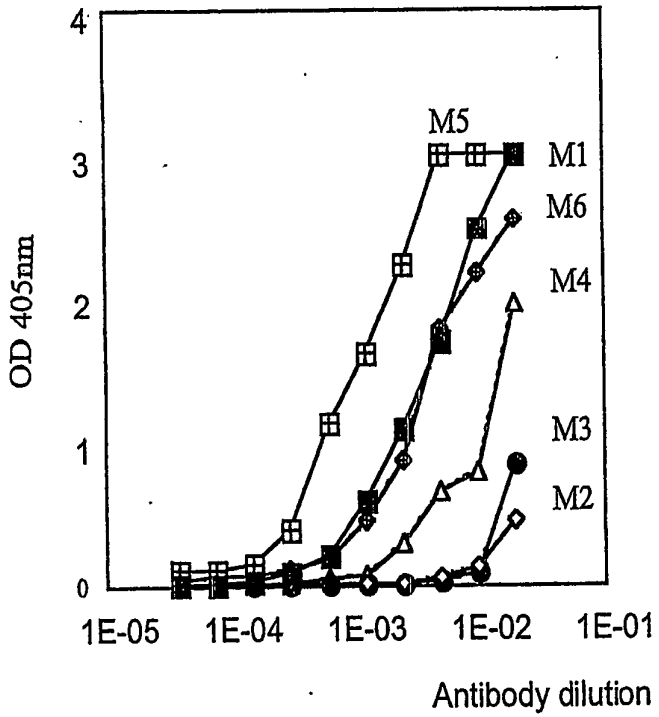

4
3
2
1
0
OD 405nm
M5
M1
M6
M4
M3
M2
1E-05
1E-04
1E-03
1E-02
1E-01
Antibody dilution

Fig. 7

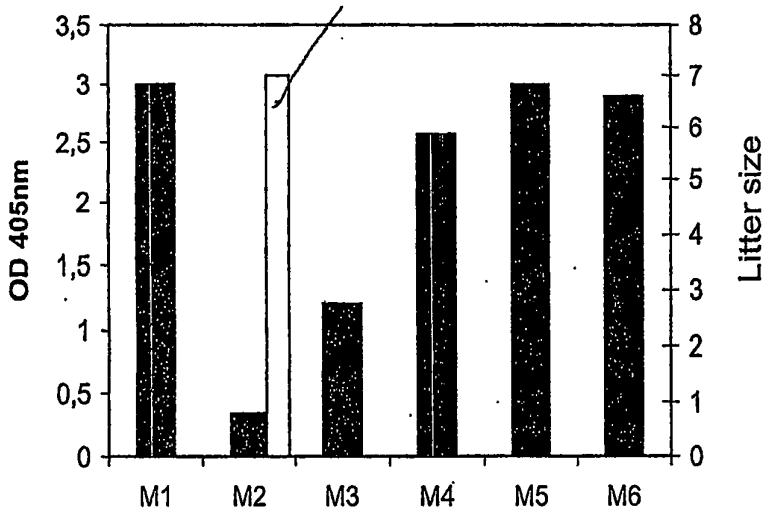

OD 405nm
3,5
3
2,5
2
1,5
1
0,5
0
Litter size
8
7
6
5
4
3
2
1
0
M1
M2
M3
M4
M5
M6

Fig. 8